ON THE SPECIFICITY OF TRYPTIC CATALYSIS

François Seydoux and Jeannine Yon

Laboratoire d'Enzymologie Physicochimique et Moléculaire

Faculté des Sciences d'Orsay - 91 - Orsay, France.

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SUMMARY. The specificity of esterasic tryptic action is discussed in terms of the formation of two intermediates (Michaelis complex and acylenzyme) during the catalytic process. From nucleophilic competition data with specific and unspecific substrates and some other complementary data, it is concluded that the tryptic specificity is essentially kinetic and exerted principally in the acylation step. Thus discrimination between specific and unspecific substrates is achieved also after the Michaelis complex formation and is not only reflected by the binding free enthalpy but also by the free enthalpy change associated with the acylation of the enzyme.

Trypsin hydrolyzes specifically peptidic, amide and ester bonds involving the carbonyl function of basic amino acids like lysine or arginine (1,2). Trypsin can also catalyse the hydrolysis of neutral aminoacid derivatives called unspecific substrates. For instance it is well known that trypsin exhibits an intrinsic chymotryptic activity toward neutral substrates like Ac-Tyr-OEt* (3). The kinetics of the esterasic tryptic action can be described in terms of the classical scheme involving two intermediate complexes : Michaelis complex (ES) and acylenzyme (ES') (4) :

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow[+P_1]{k_3} E + P_2 \qquad \text{Eq.1}$$

In this scheme K_S is the dissociation constant of ES, and k_2 and k_3 are respectively acylation and deacylation rate constants.

The purpose of the present paper is to determine to which extent the

* Aminoacids derivatives are abbreviated according to the general rules of IUPAC-IUB Commission on Biochemical Nomenclature (Biochim. Biophys. Acta 121 (1966) 1.). Arg: arginine, Cit: citrulline, Gly: glycine, Lys:lysine, Orn: ornithine, Phe: phenylalanine, Tyr: tyrosine, Ac-: acetyl, Bz-: benzoyl, To-: tosyl, -OMe: methyl ester, -OEt: ethyl ester. For example Bz-Arg-OEt is the abbreviated notation for αN-Benzoyl-L-arginine-ethyl ester. Aminoacid symbol denote the L configuration unless otherwise indicated.

enzyme exerts its specificity on each individual step defined in Eq. 1. Since it has not actually been proved that amides and peptides are hydrolysed by the same mechanism (5,6), only the case of esters will be discussed in this paper.

STEADY STATE AND INDIVIDUAL KINETIC PARAMETERS OF TRYPTIC SUBSTRATES.

Except for a few cases (7,8), tryptic hydrolyses obey the Michaelis equation and the two steady state parameters k_{cat} and K_m are related to the individual kinetic constants as follows : $k_{cat}=k_2k_3/(k_2+k_3)$, Eq. 2 and $K_m = K_Sk_3/(k_2+k_3)$, Eq. 3. These two parameters are listed in Table 1 for a series of specific and unspecific substrates. As shown in this table, specific ester substrates are distinguishable from others more by their low K_m values than by their k_{cat} values. For instance, the maximum rates of hydrolysis of Bz-Arg-OEt and Ac-Tyr-OEt are similar, but their K_m values differ by at least 4 orders of magnitude

Direct measurements by means of a stopped flow device of the K_S, k_2 and k_3 parameters are rather difficult since the acylation step has been found to be very fast. In the case of Bz-Arg-OEt, using proflavin as indicator, Bernhard and Gutfreund (12) report a k_2/k_3 ratio of about 10^3 and Himoe (13), using thionine as indicator, reports a value of 800 for the same ratio in the case of To-Arg-OMe at pH 4.8. These very high values of the k_2/k_3 ratio explain the

Table I : Steady state parameters for the tryptic hydrolysis of ester substrates.[a,b]

Substrates	k_{cat} (sec^{-1})	K_m (M)	Ref	Substrates	k_{cat} (sec^{-1})	K_m (M)	Ref
To-Arg-OMe	95	1.5×10^{-5}	c	To-Orn-OMe[e]	5.4	1.6×10^{-2}	c
To-Lys-OMe	96	4.2×10^{-5}	9	Bz-Cit-OMe[e]	0.31	4.1×10^{-2}	10
To-Lys-OEt	96.9	3.6×10^{-6}	9	Bz-Gly-OEt[e]	5.1×10^{-2}	5.6×10^{-1}	11
Bz-Arg-OEt	24	2.6×10^{-5}	c	Ac-Gly-OEt[e]	1.9×10^{-2}	8.8×10^{-2}	11
Bz-Lys-OMe	23.7	1.7×10^{-4}	9	Ac-Tyr-OEt	36	4.7×10^{-1}	c
Ac-Lys-OMe	82	2.4×10^{-4}	d	Ac-Phe-OMe	55	1.1×10^{-1}	c
Lys-OMe	6.7	2.8×10^{-4}	7	Tyr-OMe	0.7	1.8×10^{-1}	7

a: at optimum pH, 25°. b: k_{cat} data of ref 9 and 10 have been corrected on the basis of the actual tryptic specific activity toward Bz-Arg-OEt. c: this work. d : A. Dupaix, unpublished experiments. e: at pH 7.

low K_m values of specific substrates, as given by Eq.3. Evaluation of the individual parameters of Eq.1 can also be performed from steady state data. By assuming that the K_S of ester substrates is close to the K_m of the corresponding amides (14) or the K_I (inhibition constant) of the corresponding acids (15), the $k_3/(k_2+k_3)$ ratio can be obviously evaluated from the ratio K_m(ester)/K_m (amide) or K_I (acide). For instance, K_m of Bz-Arg-OEt is 2.6×10^{-6}M and that of the corresponding amide is 2.2×10^{-3}M ; the k_2/k_3 ratio is thus about 850, in agreement with the value reported in ref. 12. In addition k_{cat} values of specific substrates are independent of the nature of the leaving group, in agreement with a deacylation rate limiting step (9,16,17). In contrast, at saturation of substrate, the p-nitrophenyl ester of Ac-Gly is hydrolyzed by trypsin 19 times faster than the corresponding ethyl ester (11). Thus the limiting step of this latter neutral substrate hydrolysis appears to be acylation. In an attempt to confirm and generalize these isolated observations which indicate a different rate limiting step for specific and unspecific normal ester substrates, we have carried out nucleophilic competition experiments (18) with selected specific and unspecific substrates.

RESULTS AND DISCUSSION

Steady state data obtained for several substrates in the presence of 2M methanol and 0.6 M isopropanol are given in Table II. Isopropanol is a poor nucleophilic agent (19) and has been used to estimate possible solvent effects, since the dielectric constant of the medium is reduced from 78.5 in water to 75.7 both in 2 M methanol and 0.6 M isopropanol (20). As shown in Table II, methanol modifies differently the kinetic behavior of specific and unspecific substrates. In all cases isopropanol has only small effects on k_{cat} and K_m and therefore solvent effects can be neglected to a first approximation. K_m of specific substrates is much more increased by methanol than in the case of unspecific substrates and the opposite holds for $1/k_{cat}$. As previously discussed (18), these results indicate a deacylation rate determining step for specific substrates and, at least partly, acylation as the

Table II : Effect of methanol and isopropanol on the steady state parameters of the tryptic hydrolysis of specific and unspecific substrates.[d]

Substrates	k_{cat} (sec^{-1}) a	b	c	K_m (M) a	b	c
Bz-Arg-OEt	24	28.5	24.5	2.6×10^{-6}	4.8×10^{-6}	1.1×10^{-5}
Lys-OMe[e]	30.5±0.6	27.7±0.3	27.4±0.3	4.6±0.3 $\times10^{-3}$	4.7±0.2 $\times10^{-3}$	2.3±0.1 $\times10^{-2}$
Bz-D-Arg-OEt	9.1±0.5 $\times10^{-2}$	8.1±0.2 $\times10^{-2}$	4.5±0.2 $\times10^{-2}$	7.4±0.8 $\times10^{-4}$	1.1±0.1 $\times10^{-3}$	1.6±0.15 $\times10^{-3}$
To-Orn-OMe[f]	5.4±0.1	4.8±0.1	0.85±0.04	1.6±0.1 $\times10^{-2}$	1.2±0.1 $\times10^{-2}$	2.5±0.2 $\times10^{-2}$
Tyr-OMe[f]	2.5±0.1	2.1±0.1	0.58±0.04	4.1±0.4 $\times10^{-2}$	4.6±0.3 $\times10^{-2}$	5.1±0.7 $\times10^{-2}$
Ac-Phe-OMe[h]	53±3		11±2	1.2±0.1 $\times10^{-1}$		1.1±0.2 $\times10^{-1}$

a: without alcohol. b: 0.6 M isopropanol. c: 2 M methanol. d: at 25°, 0.025 M $CaCl_2$, pH 8. e: at pH 5.8 see ref 7. f: at pH 7. h: data for this substrate are rough estimates since its solubility is low with respect to its K_m value.

rate limiting step for unspecific substrates. In the case of Bz-Arg-OEt, k_{cat} and K_m values are both modified by methanol and therefore k_2 is close to k_3 for this substrate. Thus it is possible to calculate the individual parameters of Eq. 1 (18). We find $k_2 = 0.275\pm0.025\ sec^{-1}$, $k_3 = 0.14\pm0.01\ sec^{-1}$ and $K_S = 2.25\pm0.25\times10^{-3}$M (average of two separate experiments). A fruitful comparison of these values can be performed with those obtained in the case of the corresponding L derivative (Bz-Arg-OEt), as shown in the free enthalpy activation diagram of Fig. 1. The deacylation rate constants via ethanol, k_{-2}, which are the microscopic reverses of the acylation rate constants, k_2, for ethyl esters have been assumed in both cases to be close to the corresponding deacylation constants via water, k_3, as found with other substrates. As shown in Fig. 1, it is clearly apparent that the acylation step is more effected than the deacylation or apparent binding step by inversion of the asymmetric carbon

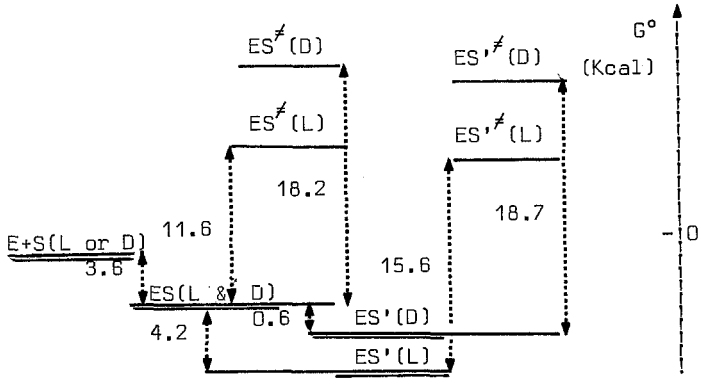

Fig. 1 : Free enthalpy activation diagram for the tryptic hydrolysis of Bz-Arg-OEt and the corresponding D derivative. ≠ refers to activated complexes and numbers indicate the values of the free enthalpy variations (Kcal/Mole).

of the substrate. It can be also noted that the negative free enthalpy change associated with the acylation step is larger in the case of the L derivative (-4.2 Kcal) than with the D derivative (-0.58 Kcal). Furthermore the acylenzyme formed with Bz-Arg-OEt appears to be more stable than for the corresponding D derivative.

Parameters of Eq. 1 for several specific and unspecific substrates are listed in Table III. The drastic decrease in the specificity index k_{cat}/K_m (4) observed when the Lys or Arg residues are replaced by other neutral or cationic residues can be especially assigned to a large reduction of k_2 rather than of k_3 or K_S. Deacylation is not a very specific step in trypsin catalysis, as already mentioned, since deacylation rate constants of neutral substrates are very close for trypsin and chymotrypsin (21) and are in some cases of the same order of magnitude as for specific substrates.

As a tentative explanation of these results, it is possible that the specific substrates are forced into a highly strained conformation in the Michaelis complex which permits a rapid acylation of the enzyme (22,23). This strain would be less pronounced in the acylenzyme, and thus the deacylation rate is slower and the acylenzyme stability greater with respect to the Michaelis complex. On the other hand, it was concluded by several authors (10,11,24) that

Table III : Individual kinetic parameters of specific and unspecific tryptic substrates.[a]

Substrates	k_{cat}/K_m (M^{-1} sec^{-1})	$K_S \times 10^3$ (M)	k_2 (sec^{-1})	k_3 (sec^{-1})
Bz-Arg-OEt	9.2×10^6	2.2[b]	2.0×10^4	24
To-Arg-OMe	6.4×10^6	7.5[b]	4.8×10^4	95
Lys-OMe[f]	2.4×10^4	14[b]	3.4×10^2	6.7
Ac-Tyr-OEt	7.6×10^2	47	36	193[c]
Ac-Phe-OMe	5.0×10^2	110	55	173[c]
To-Orn-OMe[e]	3.4×10^2	16	5.4	> 5.4
Bz-D-Arg-OEt	1.2×10^2	2.2	0.28	0.14
Tyr-OMe[f]	3.8	180	0.68	> 0.68
Ac-Gly-OEt[e]	3.6×10^{-2}	880	3.2×10^{-2}	0.6[d]

a: at optimum pH, 25°. b: K_m or K_I of corresponding amides.
c: corresponding turnover number of chymotrypsin (25) as discussed in the text. d: turnover number of the corresponding p-nitrophenyl ester (11). e: at pH 7. f: α-amino derivative (7).

neutral substrates are not bound at the same site as cationic substrates. Since, for both types of substrates, an ester bond is formed with enzyme during the acylation step, the position of neutral substrates may be closer to that of specific substrates in the acylenzyme than in the Michaelis complex, as reflected by the fact that amines activate deacylation more than acylation (11). In this manner the enzyme can realize in the acylation step a more effective discrimination between specific and unspecific substrates.

MATERIALS AND METHODS

Worthington, 2 x crystallized, Bovine trypsin, was purified by NaCl M (18). Determination of the total protein is based on an $E^{1\%}_{278\ nm}$ of 15.9. Active site titration with p-nitrophenyl p guanidinobenzoate (26) indicated a purity of about 90% with respect to the total protein concentration which is used to calculate turnover numbers. Some experiments were carried out with sulfoethyl Sephadex C 50 separated α and β trypsin (27), which showed a very similar kinetic behavior toward specific and unspecific ester substrates at optimum pH*.

* : G. Foucault, unpublished experiments in this laboratory.

Substrates are commercial products of the best available grade. Alcohols were redistilled freshly before use.

Kinetic measurements were carried out using a pH stat arrangement as previously described (14). K_m determination in the case of Bz-Arg-OEt and To-Arg-OMe were performed by means of the integrated Michaelis equation by following the change in OD with $\Delta E^M_{252\ nm} = 1100$ and $\Delta E^M_{245\ nm} = 600$ respectively on the expanded scale (2.5×10^{-2} OD, full deviation) of a Cary 16K spectrophotometer. In all cases, the rate of release of the P_2 product (acid) was measured in 0.025 M, $CaCl_2$, at 25°. Statistical analysis of the data was performed according to the iterative procedure of Cleland (28), programmed for a Wang electronic calculator.

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